

NEW IMMUNOPROTECTIVE INFLUENZA ANTIGEN AND  
ITS USE IN VACCINATION

The present invention relates to new immunoprotective influenza antigens, which are non-existent in nature. The invention further relates to the use of the antigens for vaccination and to vaccines containing them, as well as to methods for preparing the antigens.

BACKGROUND OF THE INVENTION

Influenza is caused by an RNA virus of the myxovirus group. Influenza viruses can be classified into three types (A, B and C), based on antigenic differences in the nucleoprotein and the matrix protein. Type A and B influenza viruses each contain 8 RNA segments, while type C only has 7 RNA segments. Influenza A is most important and is very pathogenic for man, as well as for animals, for example pigs and horses. Type B influenza causes disease in humans. Influenza C is less severe and has been isolated from humans and pigs. The virus is transmitted through the air, mainly in droplets expelled during coughing and sneezing. The influenza viruses cause an infection of the respiratory tract, that is usually accompanied with coughing, high fever and myalgia. Although an influenza infection does not often lead to the death of the infected individual, the morbidity can be severe. As a consequence thereof influenza epidemics may lead to substantial economic loss. Furthermore, influenza infection can be more dangerous for certain groups of individuals, such as those having suffered from a heart attack, CARA patients or the elderly. A vaccine against influenza is therefore highly desirable.

The influenza A virus contains in its membrane two highly immunogenic, but very variable proteins, the hemagglutinin and the neuraminidase. Due to the variability of these two proteins a broad spectrum, long lasting vaccine against influenza A has so far not been developed. The influenza vaccine commonly used, has to be adapted almost every year to follow the antigenic drift of the virus. In these circumstances the vaccine can protect about 80% of the immunized persons. When more

drastic changes occur in the virus, known as antigenic shift, the vaccine is no longer protective.

SUMMARY OF THE INVENTION

It is therefore the object of the present invention to provide a new immunoprotective antigen for use in vaccines which is not based on the rapidly changing hemagglutinin and/or neuraminidase and which therefore lacks the disadvantages of these known antigens and vaccines based thereon.

In the research that led to the present invention it was found that well conserved membrane proteins of influenza other than hemagglutinin and neuraminidase can be used for eliciting protection. Particularly useful for this approach is the membrane protein M2.

DETAILED DESCRIPTION OF THE INVENTION

M2 mRNA is encoded by RNA segment 7 of the influenza A virus. It is encoded by a spliced mRNA (Lamb et al., 1981). Like the hemagglutinin and the neuraminidase, the M2 protein is an integral membrane protein of the influenza A virus. But the protein is much smaller, only 97 amino acids long. 24 amino acids at the amino terminus are exposed outside the membrane surface, 19 amino acids span the lipid bilayer, while the remaining 54 residues are located on the cytoplasmic side of the membrane (Lamb et al., 1985).

The M2 protein is abundantly expressed at the cell surface of influenza A infected cells (Lamb et al., 1985). The protein is also found in the membrane of the virus particle itself, but in much smaller quantities, 14 to 68 molecules of M2 per virion (Zebedee and Lamb, 1988). The M2 protein is posttranslationally modified by the addition of a palmitic acid on cysteine at position 50 (Sugrue et al., 1990).

The M2 protein is a homotetramer composed of two disulfide-linked dimers, which are held together by noncovalent interactions (Sugrue and Hay, 1991). By site-directed mutagenesis, Holsinger and Lamb (1991) demonstrated that the cysteine residue at position 17 and 19 are involved in disulfide bridge formation. Only

ins  
aa  
a  
000000-94080400

5 disulfide bridge is formed in the same dimer (already linked by Cys 17 - Cys 17) or with the other dimer.

15 Two virus strains do not fit in this conserved pattern, A/PR/8/34 (H1N1), which shows one amino acid change, and A/Fort Monmouth/1/47 (H1N1), which shows three amino acid differences. These two strains probably represent side branches in the evolutionary tree.

25 A/Singapore/1/57 and A/Port Chalmers/1/73 (all described  
by Zebedee and Lamb (1989)), A/Udorn/72 (Lamb and Lai  
(1981)), A/Leningrad/134/57 (Klimov et al. (1992)), A/Ann  
Arbor/6/60 (Cox et al. (1988)), A/Bangkok/1/79 (Ortin et  
al. (1983)), A/New York/83 (Belshe et al. (1988)), A/Fort  
30 Monmouth/1/47 (EMBL U02084), A/USSR/90/77 (EMBL X53029)  
and A/Guangdong/39/89 (EMBL L 18999).

Table 1

# Amino acid sequence of the extracellular domain of the M2 protein

Strain	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Q AWS/33 (H1N1)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp <sup>A</sup>
Q AWSN/33 (H1N1)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp <sup>A</sup>
Q APR/8/34 (H1N1)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Gly	Ser	Ser	Asp
Q A/Fort Monmouth/1/47 (H1N1)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Thr	Lys	Asn	Glu	Trp	Glu	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp <sup>A</sup>
Q A/Fort Warren/1/50 (H1N1)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp <sup>A</sup>
Q AUSSR/90/77 (H1N1)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp <sup>A</sup>
Q A/Singapore/1/57 (H2N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp <sup>A</sup>
Q A/Leningrad/134/57 (H2N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp <sup>A</sup>
Q A/Ann Arbor/6/60 (H2N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp <sup>A</sup>
Q AUdom/72 (H3N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp <sup>A</sup>
Q A/Port Chalmers/1/73 (H3N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp <sup>A</sup>
Q A/Bangkok/1/79 (H3N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp <sup>A</sup>
Q ANY/83 (H3N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp <sup>A</sup>
Q A/Guangdong/39/89 (H3N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp <sup>A</sup>

It was anticipated by the present inventors that the conserved character of this type of membrane proteins could make them good candidates for vaccine development. In principle, the protective capacity of anti-M2 antibodies is already known. Experimental data demonstrated that a monoclonal antibody directed against the extracellular part of the M2 protein (14C2) can diminish the spread of the virus, although the infectivity of the virus in vitro was not reduced (Zebedee and Lamb, 1988). Furthermore it was demonstrated that passively administered monoclonal antibody (14C2) could inhibit viral multiplication in the lungs of mice (Treanor et al., 1990). Both approaches rely on the administration of anti-M2 antibodies. However, the passive administration of monoclonal antibodies as a means of defense against infection is preferably avoided because of the immunogenicity of heterologous immunoglobulins which, upon repeated administration, can lead to the clearing of the antibodies from the body and thus to a reduction of the efficacy of the treatment. Even homologous antibodies can elicit anti-idiotypic antibodies. Furthermore, it was found that humans infected with the virus do have anti-M2 antibodies but these do not protect against infection, (either their concentration or their nature are not sufficient to confer efficacy). This makes it unlikely that passive administration of anti-M2 antibodies is suitable for use in humans. It also teaches away from trying to develop vaccines for humans based on this antigen.

Recently, protection of mice against an infection with homologous or heterologous virus was described (Slepishkin et al., 1995). These authors used a formulation of incomplete Freund's adjuvant and a membrane extract of Sf9 cells expressing the complete M2 protein for immunizations. However, this approach is also not suitable for vaccination of humans because it relies on the use of the exceptionally potent Freund's adjuvant which is prohibited in humans.

In summary, use of antibodies for providing protection against influenza is preferably to be avoided. Moreover, it is unlikely that prophylactic treatment with antibodies will be effective in humans. Immunization with complete M2 protein in humans as described is not realistic because it relies on incomplete Freund's adjuvant which cannot be used in humans, and is counter-indicated in higher animals.

It is thus the object of the present invention to provide for an alternative influenza antigen that is sufficiently immunoprotective against a broad spectrum of influenza strains and is not dependent on Freund's adjuvant, such that it can be used in human beings.

According to the invention it has now been found that it is possible to prepare such a novel antigen that does not exist in nature. For this the extracellular part of a conserved influenza membrane protein or a functional fragment thereof is fused to a presenting carrier, for example a (poly)peptide. The conserved influenza membrane protein is for example the well conserved, extracellular part of the M2 protein. The membrane protein is preferably genetically fused to a presenting (poly)peptide as the presenting carrier, which (poly)peptide stabilizes the extracellular part and surprisingly potentiates the immunogenicity of the fusion product thus obtained. It is thought that the presenting (poly)peptide brings the extracellular part into its wild type structure, thus presenting the antigen in a form that is also found on the virus and on the infected cells.

A 'functional fragment of the conserved influenza membrane protein' is a fragment that is capable of eliciting a statistically significant higher immunoprotection when administered in an immunoprotective dose to test members of a species than is found in control members of the same species not receiving the functional fragment.

5 protein in viral particles and on infected cells, where the free N-terminus extends in the extracellular environment, is mimicked.

10 toxin fragment C or yeast Ty particles. 'Presenting (poly)peptides' are intended to encompass every stretch of amino acid(s) that can present the extracellular part, in a substantially wild type form, towards the environment.

15 Alternatively, the presenting carrier can be a non-peptidic structure, such as glycans, polyethylene glycols, peptide mimetics, synthetic polymers, etc..

After expression of the novel antigen in a suitable acceptor cell, it can be used either as such (depending on the acceptor cell), as part of a membrane fragment or in isolated form.

The term 'presenting carrier' is used to indicate all types of presenting molecule, both (poly)peptides and others.

25 It will be clear for the person skilled in the art that a gene construct, comprising the coding information for the antigen and the presenting (poly)peptide, can not only be used to prepare the new antigen, as described above, but that it can also be  
30 used, optionally in the presence of suitable transcription and/or translation regulatory sequences, in a DNA vaccine, or in vaccinia based vaccine constructions.

A presenting (poly)peptide can be incorporated  
35 into the fusion product in a single copy or in multiple  
copies. The third complement protein fragment d (C3d) is  
preferably used in more copies, preferably 3 or more.

**NOTES**

In a preferred embodiment of the invention the fusion product further may comprise an additional peptide at an appropriate internal site (Schödel et al., 1992) or C-terminal (Borisova et al., 1989). This additional peptide is intended to further increase the protective capacity of the antigen, and may for example be a T helper cell epitope or a cytotoxic T cell epitope.

The antigen of the invention is obtainable by preparing a gene construct comprising a coding sequence for at least the extracellular part of a conserved influenza membrane protein or a functional fragment thereof and optionally the coding sequence for a presenting (poly)peptide operably linked thereto, optionally in the presence of suitable transcription and/or translation and/or secretion regulatory sequences, bringing this gene construct in a suitable acceptor cell, effecting expression of the gene construct in the acceptor cell and optionally isolating the antigen from the acceptor cell or its culture medium.

The requirement for transcription and/or translation and/or secretion regulatory sequences depends on whether the gene is to be integrated into a vector or whether integration in the genome of the acceptor cell is at a position already providing these signals.

The coding sequence for a presenting (poly)peptide is only present when the fusion product is a fusion between the antigen and a peptidic structure and if it is desirable to directly link the two structures in the DNA construct. In all other instances, the presenting carrier may be added to the antigen in a different manner.

The suitable acceptor cell can be selected for example, from E. coli, Lactococcus lactis, Lactobacillus plantarum, yeast (e.g. Pichia pastoris), insect cells (e.g. Sf9), mammalian cells (e.g. Vero cells) and the like. In the case of L. lactis the antigen need not be isolated but the engineered bacteria can be used directly for intranasal or oral use.

00000-908000



The invention further relates to vaccines that comprise at least the antigen of the invention. This antigen can be in isolated form or being part of a membrane fragment or being expressed on the acceptor  
5 cell. The antigen of the invention can be used together with suitable excipients. The person skilled in the art of vaccine design will be capable of selecting suitable excipients. Guidance may for example be found in Methods in molecular medicine: Vaccine Protocols (1996). Eds.  
10 Robinson, A., Farrar, G.H. and Wiblin, C.N. Humana Press, Totowa, New Jersey, USA.

The antigens of the invention may be used alone or in combination with one or more other influenza antigens, such as neuraminidase, hemagglutinin or native  
15 M2.

Furthermore, the invention relates to the use of the antigens in the preparation of a vaccine against influenza. The vaccines can be direct vaccines, i.e. vaccines containing the fusion products or indirect, DNA  
20 vaccines. The latter are vaccines, comprising the fusion cDNA under the regulation of a eukaryotic promoter that can function in the recipient. The actual antigen is then produced in the recipient of the vaccine.

The vaccines of the invention are intended both  
25 for use in humans and in animals, for example pigs and horses of which it is known that they are infected by influenza A.

A similar approach as described here for preparing novel fusion antigens of influenza A can be  
30 adopted to prepare similar fusion antigens and vaccines containing the fusion antigens or DNA encoding the fusion antigens for influenza B and C.

The invention also relates to a method of preparing the antigens, comprising the steps of:

35 a) preparing a gene construct comprising a coding sequence for at least the extracellular part of a conserved influenza membrane protein or a functional fragment thereof and at least one coding sequence for a

00000-9108600

presenting (poly)peptide operably linked thereto,  
optionally in the presence of suitable transcription  
and/or translation and/or secretion regulatory sequences,

b) bringing this gene construct in a suitable  
5 acceptor cell,

c) effecting expression of the gene construct in the acceptor cell, and

d) optionally isolating the antigen from the acceptor cell or its culture medium.

The invention will be further illustrated by the following example, that is in no way intended to limit the invention. The example describes in detail the preparation of fusion proteins of M2 sequence with various presenting (poly)peptides and the use thereof in immunization. Instead of M2 and the presenting carriers described here, the skilled person will be capable of choosing another conserved influenza membrane protein and other presenting carriers.

~~In the example reference is made to the~~  
~~20 following figures:~~

BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1 : Construction of pATIPM2m1.**

E1 and E2 = first and second exon of the influenza M2 protein,

M2e = extracellular part of the M2 protein,

25 M2t = transmembrane part; and

M2c = cytoplasmic tail.

Bold line = vector.

(a) removal of the intron out of the m2 gene,

(b) introduction of a BclI site between the extracellular part and the transmembrane domain of the M2 protein,

(c) nucleotide and amino acid sequence of the extracellular part of the M2 protein of A/PR/8/34.

Figure 2 : Construction of pIPM2hB2Mm2s2

35 ori = origin of replication,

cat = chloramphenicol acetyltransferase,

bla =  $\beta$ -lactamase,

lpp = lipoprotein,

$a^2$  $a^2$

MW = molecular weight marker,

NI = not induced culture,

I = induced culture.

**Figure 6** : Sequence of the amino terminus of the M2 protein compared to the amino terminus of IPM2HBcm, as experimentally determined. Sequence of A/Udorn/72 (Lamb and Zebedee, 1985).

**Figure 7** : Soluble fractions of strain MC1061[pcl857] transformed with pPLc245 (control), pPLcA 1 (expression of HBc) or pPLcIPM2HBcm (expression of IPM2HBcm), respectively, analyzed in a native state by means of a dot blot. Detection with (A) a monoclonal antibody against HBc and (B) a monoclonal antibody specific for the extracellular part of the M2 protein.

NI = not induced culture,  
I = induced culture.

**Figure 8** : Overview of (A1) rectal temperature, (A2) weight and (B) survival of the mice vaccinated with IPM2HBcm after a lethal challenge with 5 LD<sub>50</sub> m.a. A/PR/8/34. The statistical significance was calculated by the Fisher's exact test. Mice immunized with different doses of antigen were compared to the control group. The following results were obtained: for 50 µg IPM2HBcm p<0.001; for 10 µg p<0.005 and for the 5 µg dose p<0.05.

Figure 8C shows the survival of the mice vaccinated intraperitoneally with IPM2HBcm, and IM2HBcm, respectively, after a lethal challenge with 30 HAU X-47. Figure 8D shows the survival of the mice vaccinated intranasally with IPM2HBcm, and IM2HBcm, respectively, after a lethal challenge with 30 HAU X-47.

**Figure 9** : Analysis of the serum samples of the four set ups reported in figure 8. The pre-immune serum (a), the serum taken after the first (b), after the second (c) and after the third (d) immunization and the serum taken after challenge (e) were initially diluted 1/50. The consecutive dilution steps were 1/3. The plotted absorbance is a corrected value obtained as described in Results, Analysis of the serum samples.

**Figure 10 : Construction of pPLcIM2HBcm.**

ori = origin of replication,

cat = chloramphenicol acetyltransferase,

bla =  $\beta$ -lactamase,

5 M2e = extracellular part of the M2 protein,

HBc = hepatitis B core.

**Figure 11 : Analysis of the soluble fraction,**  
containing 5  $\mu$ g HBc or I(P)M2HBcm (as determined in an  
ELISA (see Materials and methods)), of strain MC1061

10 [pcI857] containing respectively the plasmids pPLc245

(control), pPLcA1 (expression of HBc), pPLcIPM2HBcm

(expression of the fusion protein IPM2HBcm with the  
extracellular part of the M2 protein derived from

A/PR/8/34) or pPLcIM2HBcm (expression of IM2HBcm,

15 containing the more universal M2 sequence) on a SDS 12.5%  
PAGE-gel.

MW = molecular weight marker,

NI = not induced,

I = induced culture.

20 **Figure 12 : Analysis of the soluble fraction,**  
containing 2.5  $\mu$ g HBc or I(P)M2HBcm (as determined in an  
ELISA (see Materials and methods)), of strain MC1061

[pcI857] containing respectively the plasmids pPLc245

(control), pPLcA1 (expression of HBc), pPLcIPM2HBcm

25 (expression of IPM2HBcm) or pPLcIM2HBcm (expression of  
IM2HBcm) on a Western blot (see Materials and methods).

Detection with (A) a monoclonal antibody directed against

HBc and (B) a monoclonal antibody specific for the

extracellular part of the M2 protein.

30 MW = molecular weight marker,

NI = not induced,

I = induced culture.

**Figure 13 : Overview of the oligonucleotides**  
used for PCR amplification of hbc and i(p)m2hbc. 's' or

35 'a' following the name of the oligonucleotide stands for  
the use of these primers in the sense (s) or anti-sense  
(a) orientation. The boxed sequence indicates the changed  
Leu codons.

**Figure 14** : Overview of the construction of hbc and m2hbc fusions in vectors for L. lactis.

ori = origin of replication for E. coli,

ori(+) = origin of replication for L. lactis,

ermA and ermM = erythromycin resistance genes,

P1 = L. lactis promoter,

bla =  $\beta$ -lactamase,

HBc = hepatitis B core,

M2e = extracellular part of the M2 protein,

usp45-ss = signal sequence of usp45,

mIL2 = murine interleukin 2 and

mIL6 = murine interleukin 6.

**Figure 15** : Analysis of the expression of

Hepatitis B core (HBc) and M2-HBc fusion proteins in a

Western blot. An equivalent of  $10^9$  L. lactis bacteria of strain MG1363 containing respectively pTrex1 (control), pT1HBc, pT1HBcIL2, pT1HBcIL6 (expression of HBc alone or in combination with mIL2 or mIL6, respectively),

pT1PM2HBc, pT1PM2HBcIL2, pT1PM2HBcIL6 (expression of IPM2HBcm alone or in combination with mIL2 or mIL6, respectively), pT1M2HBc, pT1M2HBcIL2, pT1M2HBcIL6 (expression of IM2HBcm alone or in combination with mIL2 or mIL6, respectively), was analyzed in a SDS 12.5% PAGE-gel. The first antibody, p-anti-HBc (Dako Corporation, Carpinteria, CA., USA) was diluted 5000 times. The bound antibodies were detected with a 1/2000 dilution of the polyclonal anti-rabbit IgG labeled with alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL., USA). I(P)M2HBc stands for either IPM2HBcm or IM2HBcm.

MW = molecular weight marker,

C = control and

- = expression of the antigen alone.

**Figure 16** : Analysis of the expression of M2-

HBc fusion proteins in a Western blot. An equivalent of 2 to  $3 \times 10^9$  L. lactis bacteria of strain MG1363 containing respectively pT1HBc (control), pT1PM2HBc, pT1PM2LHBc (expression of IPM2HBcm), pT1M2HBc, pT1M2LHBc (expression

004020-91036100

of IM2HBcm), was separated on a SDS 12.5% PAGE-gel. The fusion proteins were detected with an IgG fraction of a polyclonal mouse anti-M2e antibody (see Materials and methods). The bound antibodies were detected with a  
 5 1/2000 dilution of the alkaline phosphatase conjugated polyclonal anti-mouse IgG ( $\gamma$ -chain specific) (Southern Biotechnology Associates, Birmingham, AL., USA).

MW= molecular weight marker,

C = control,

10 E = leucine codons optimal for use in E. coli, and

L = leucine codons optimal for use in L. lactis.

These are the plasmids pT1PM2LHBc and pT1M2LHBc, respectively. I(P)M2HBc stands for either IPM2HBcm or IM2HBcm.

15 **Figure 17** : Overview of the oligonucleotides used for PCR amplification of the extracellular part of the M2 protein and C3d.

's' or 'a' following the code name of the oligonucleotide stands for the use of these primers in the sense (s) or  
 20 anti-sense (a) orientation. The boxed region indicates the changed Leu codons.

**Figure 18** : Overview of the construction of m2c3d3 fusions in L. lactis.

ori = origin of replication for E. coli,

25 ori(+) = origin of replication for L. lactis,

ermA and ermM = erythromycin resistance genes,

P1 = L. lactis promoter,

bla =  $\beta$ -lactamase,

M2e = extracellular part of the M2 protein,

30 usp45-ss = signal sequence of usp45,

spaX = anchor sequence derived from Staphylococcus aureus protein A,

C3d = complement protein 3 fragment d, and

mIL6 = murine interleukin 6.

35 **Figure 19** : Overview of the oligonucleotides used for PCR amplification of ttfc and m2ttfc.

's' or 'a' following the name of the oligonucleotide stands for the use of these primers in the sense (s) or

004020-91036p00

anti-sense (a) orientation. The boxed region indicates the changed Leu codons.

**Figure 20** : Overview of the construction of m2ttfc in vectors for L. lactis.

5 ori = origin of replication for E. coli,  
ori(+) = origin of replication for L. lactis,  
ermM and erm $\mu$  = erythromycin resistance genes,  
P1 = L. lactis promoter,  
bla =  $\beta$ -lactamase,

10 TTFC = tetanus toxin fragment C,  
M2e = extracellular part of the M2 protein,  
usp45-ss = signal sequence of usp45,  
mIL2 = murine interleukin 2, and  
mIL6 = murine interleukin 6.

15 **Figure 21** : Analysis of the expression of IPM2TTFC fusion protein in a Western blot. An equivalent of 10<sup>9</sup> L. lactis bacteria of strain MG1363 containing respectively pT1TT (control), pT1PM2LTT (expression of IPM2TT), pT1PM2LTTIL2 (expression of IPM2TT in

20 combination with mIL2) or pT1PM2LTTIL6 (expression of IPM2TT in combination with mIL6), was analyzed in a SDS 10% PAGE-gel. The first antibody, an IgG fraction of a polyclonal mouse anti-M2e antibody (see Materials and methods) was diluted 2500 times. The bound antibodies were detected with a 1/2000 dilution of the polyclonal anti-mouse IgG labeled with horseradish peroxidase (Southern Biotechnology Associates, Birmingham, AL., USA). 30 mg 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, Mo., USA), was dissolved in 10 ml methanol.

30 Afterwards 40 ml PBS, pH 7.4 and 150  $\mu$ l H<sub>2</sub>O<sub>2</sub> was added.  
MW = molecular weight marker,

- = expression of the antigen alone,

mIL2 = expression of the antigen in combination with mIL2,

35 mIL6 = expression of the antigen in combination with mIL6.

a 000000-910354002



[illegible][illegible][illegible][illegible][illegible]

**W**

[illegible]

**W**

[illegible]

**W**

**W**

[illegible]

**W**

[illegible][illegible][illegible][illegible][illegible][illegible][illegible][illegible]

**W**

[illegible]

vaccinated with  $3 \times 10^6$   $\mu$ g IM2HBcm are compared with passively immunized mice (P).

**Figure 29** : Overview of the DNA vaccination constructs.

- 5 RT = reverse transcriptase  
 PCMV = cytomegalovirus promoter  
 bla =  $\beta$ -lactamase  
 npt = neomycin resistance.

**Figure 30** : Expression in HEKT cells analyzed on a Western blot. The first antibody (paM2 (see Materials and Methods)) was diluted 2000 times. The bound anti-M2 antibodies were detected with an alkaline phosphatase labelled anti-mouse IgG.

MW = molecular weight marker

- 15 M2 = M2 protein expressed in insect cells  
 1 = pCDNA3  
 2 = pCIM2  
 3 = pCIM2HBcm  
 4 = pCIP3M2HBcm.

20 **Figure 31** : Antibody response against the M2 protein analyzed in an ELISA.

A. Microtiterplates were coated with periplasm containing hB2M or IPM2hB2M respectively (see Materials and Methods).

25 B. Microtiterplates coated with M2 protein expressed in insect cells (see Materials and Methods).

The following abbreviations will be used:

- 1 LD<sub>50</sub> : lethal dose, the viral challenge required  
 30 to kill half of the population of infected mice  
 BCIP : 5-bromo-4-chloro-3-indolylphosphate  
 bp : base pair(s)  
 CIP : calf intestine phosphatase  
 35 C3d : complement protein 3 fragment d  
 DEA : diethylamine  
 HAU : hemagglutination units  
 hB2M : human  $\beta$ 2-microglobulin

	HbC	:	Hepatitis B core protein
	IM2HBcm	:	universal influenza A M2 protein fragment fused to HbC
5	IPM2hB2Mm	:	influenza A M2 protein fragment (from A/PR/8/34) fused to hB2M
	IPM2HBc	:	influenza A M2 protein fragment (from A/PR/8/34), fused to HbC, containing four additional amino acids between the first methionine and the start of the
10			extracellular part of the M2 protein
	IPM2HBcm	:	influenza A M2 protein fragment (from A/PR/8/34) fused to HbC
	IPTG	:	isopropyl- $\beta$ -D-thiogalactoside
	m.a.	:	mouse adapted
15	M2C3d3	:	universal influenza M2 fragment fused to three copies of C3d
	cm2C3d3	:	cytoplasmic form of M2C3d3
	sm2C3d3	:	secreted form of M2C3d3
	sm2C3d3X	:	form of M2C3d3 covalently attached to the
20			cell wall
	MES	:	2-(N-morpholino)ethanesulphonic acid
	MPLA	:	monophosphoryl lipid A
	NBT	:	nitro blue tetrazolium
	OmpA-ss	:	signal sequence of the outer membrane
25			protein A
	PCR	:	polymerase chain reaction
	SDS-PAGE	:	sodium dodecylsulfate polyacrylamide gel electrophoresis
	TDM	:	trehalose dicorynomycolate
30	phP	:	baculovirus polyhedrin promoter
	sgp67	:	secretion signal of the baculovirus gp67 protein

### 35 EXAMPLE

#### INTRODUCTION

This example demonstrates the preparation of various fusion antigens based on the influenza A virus M2

00100-34036160

protein. The M2 fragment was fused to the amino terminus of various presenting carriers.

## MATERIALS AND METHODS

### 1. Bacterial strains and plasmids

All plasmid constructions, made for expression in Escherichia coli, were performed in strain MC 1061 (hsdR mcrB araD139 $\Delta$ (araABC-leu)7697  $\Delta$ lacX74 galU galK rpsL thi (Casadaban and Cohen, 1980) because of high efficiency of transformation. The first transformation after mutagenesis was performed in WK6 $\lambda$ mutS ( $\Delta$ (lac-proAB), galE, strA, mutS::Tn10/lacI<sup>r</sup>, ZAM15, proA'B'; Zell and Fritz, 1987). Expression studies of human  $\beta_2$ -microglobulin and derivatives were performed in E. coli strain C3000 (Hfr, sup<sup>+</sup>, thi( $\lambda^-$ )). Expression studies of the Hepatitis B core protein and derivatives were carried out in MC1061 [pcI857].

pcI857 was described in Remaut et al., 1983b. A derivative of this plasmid pcI857K1 was described in Steidler et al., 1994.

The plasmid p714 (Parker and Wiley, 1989) was a kind gift of Dr. K. Parker and the plasmid pPLcA1 (Nassal, 1988) of Dr. M. Nassal. The plasmid pPLc245 was described in Remaut et al., 1983a.

For the constructions and expressions in Lactococcus lactis strain MG1363 (Gasson, 1983) was used. The vector for constitutive expression in L. lactis, pTREX1 (Wells and Schofield, 1996) was a generous gift from Dr. K. Schofield. The plasmid pL2MIL2, for the expression of interleukin 2, is described in Steidler et al., 1995. An analogous plasmid for the expression of interleukin 6, pL2MIL6, is described in Steidler et al., 1996.

The vector pSG5.C3d.YL (Dempsey et al., 1996) is a gift from Dr. Fearon.

The baculovirus transfer vector pACGP67A (PharMingen, San Diego, CA, USA) contains a modified segment of the baculovirus genome, including the

polyhedrin promoter followed by the secretion signal derived from the gp67 baculovirus protein and a cloning site for the insertion of a foreign gene sequence. It is constructed to allow integration into the baculovirus genome (or modified version thereof) by homologous recombination. The resulting recombinant baculovirus is capable of expressing the gene of interest from the polyhedrin promoter as a secreted protein by cleavage of the gp67 secretion signal.

10

## 2. Virus

Influenza virus A/PR/8/34 (H1N1) was adapted to mice by several lung passages. After adaptation, the virus was grown in eggs (Kendal et al, 1982) and purified over a sucrose gradient. The titer [(hemagglutination units (HAU) (Hirst, 1941; Kendal et al, 1982)] and the lethality in mice were determined. For m. a. A/PR/8/34, 1 LD<sub>50</sub> corresponded to 10 HAU present in 50 µl.

Influenza strain X-47 (H3N2) (Baez et al., 1980) was used in experiments for heterologous challenge. This strain was adapted to mice by several lung passages.

## 3. Animals

Female Balb/c mice were purchased from Charles River Wiga (Sulzfeld, Germany). The mice were used at the age of 6 to 7 weeks.

## 4. Antibodies

The monoclonal mouse antibody directed to the Hepatitis B core protein was a kind gift from Dr. Sc. H. Claeys (Bloedtransfusiecentrum, Leuven).

A mouse monoclonal antibody specific for the human  $\beta_2$ -microglobulin was purchased from Boehringer (Mannheim, Germany).

Alkaline phosphatase conjugated antibodies specific for mouse IgG or mouse IgG ( $\gamma$  chain specific) were bought from Sigma Chemical Co. (St. Louis, Mo., USA).

004020-51030160

### 5. Growth media

E. coli was grown in LB medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl) unless mentioned otherwise. The minimal M9 medium (Miller, 1972), supplemented with 0.2% casamino acids, was used in experiments when the expressed proteins were secreted into the growth medium and had to be purified.

M17 growth medium (Difco Laboratories, Detroit, MI, USA) supplemented with 0.5% glucose (GM 17) was used for culturing L. lactis. Erythromycin was used at a concentration of 5 µg/ml (medium GM17E). L. lactis was grown at 28°C without shaking.

The hybridomas and the myeloma cells were grown in RPMI 1640 (Gibco BRL, Bethesda, Md., USA) supplemented with 10% fetal calf serum, 0.3 mg/ml L-glutamine, 0.4 mM sodium pyruvate, 100 u/ml penicillin and 100 ng/ml streptomycin.

Sf9 insect cells were grown in TC100 medium (Gibco BRL, Bethesda, MD, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 ng/ml streptomycin.

### 6. Adjuvants

For the first immunization Ribi adjuvant (Ribi Immunochem Research Inc., Hamilton, MT, USA) was used. A complete dose of Ribi adjuvant contains 50 µg MPLA (monophosphoryl lipid A), 50 µg TDM (trehalose dicorynomycolate), 2% squalene and 0.01% Tween 80.

For the second and third immunization MPLA (Ribi Immunochem Research Inc., Hamilton, MT, USA) was used alone or mixed with an equal quantity of adjuvant peptide (Sigma Chemical Co., St. Louis, Mo., USA).

### 7. DNA manipulations

Restriction enzymes, DNA polymerases, T4 polynucleotide kinase and T4 DNA ligase (Boehringer, Mannheim, Germany; Gibco BRL, Bethesda, Md. USA, or New England Biolabs, Beverly, MA, USA) were used as

001020-9086160

recommended by the manufacturer. For analytical purposes, plasmid DNA was extracted according to Birnboim and Doly (1979). For preparative purposes, plasmid DNA was isolated according to Kahn et al. (1979). Restriction fragments of DNA were isolated by the Geneclean method according to Vogelstein and Gillespie (1979) and Struhl (1985). The required materials were purchased from Bio 101 (La Jolla, CA., USA). For the isolation of plasmid DNA out of L. lactis, a pretreatment of the bacteria is necessary to weaken the cell wall. The bacterial pellet was resuspended in 50  $\mu$ l TE (10 mM Tris-HCl pH 8 - 1 mM EDTA). Afterwards, another 50  $\mu$ l TE, supplemented with 10 mg/ml lysozyme (Boehringer, Mannheim, Germany) and 200 u/ml mutanolysin (Sigma Chemical Co., St. Louis, Mo., USA) was added. This mixture was incubated for 10 min at 37°C and then put on ice for 5 min. Further treatments were identical to those used for plasmid isolation from E. coli.

For all constructions in L. lactis purified plasmid DNA (Qiagen, Hilden, Germany) was used. The DNA fragments were purified from agarose gels by using Qiaex II (Qiagen, Hilden, Germany).

#### 8. PCR amplification

All PCR reactions were carried out following a basic protocol. In each reaction about 50 ng pure template and 50 pmol sense and anti-sense oligonucleotides (Life Technologies, Paisley, UK) were used. Two units Vent<sub>R</sub><sup>®</sup> DNA polymerase (New England Biolabs, Beverly, MA., USA) were added after heating of the samples to 94°C. The annealing temperature ( $T_a$ ) was set, according to the composition of the primer, at about 7°C below the melting temperature ( $T_m$ ). In these PCR amplifications the best results were obtained at 60°C. The synthesis of hbc and the fusion genes ipm2hbc and im2hbc, was carried out for 45 seconds at 72°C. The synthesis of the sequence, coding for the extracellular part of the M2 protein (cm2 and sm2), was left for 20

seconds at 72°C. A total of thirty amplification rounds were performed. The control reactions did not contain oligonucleotides. Three different concentration of  $\text{MgSO}_4$  were used, 2, 3 and 4 mM. The PCR reaction that produced a significant amount of the expected fragment under the most stringent conditions (lowest  $\text{Mg}^{2+}$  concentration and highest  $T_m$ ) was used for further cloning.

The C3d3 fragment was amplified from pSG5.C3d.YL with the oligonucleotides C3ds and C3da using Pwo DNA Polymerase (Boehringer, Mannheim, Germany). The annealing temperature was set at 60°C and the synthesis was performed for 2 min at 72°C.

Amplification of the baculovirus gp67 secretion signal was done with Taq polymerase (Boehringer Mannheim, Germany) from pACGP67A using the primers GP67s en GP67a. A total of 25 cycli were performed with synthesis at 72°C for 1 min.

#### 9. Ligation

The ligations for L. lactis were performed with Ready-To-Go™ T4 DNA Ligase (Pharmacia Biotech, Uppsala, Sweden). After incubation for 1h at 20°C, the mixture was extracted with phenol (Life Technologies, Paisley, UK) and chloroform/iso-amyl alcohol (24/1). The DNA was precipitated with see-DNA (Amersham International, Buckinghamshire, UK). The complete resuspended pellet was used for electroporation (Wells et al., 1993).

#### 10. Protein purification media

All chromatography media were purchased from Pharmacia Biotech (Uppsala, Sweden), except CF11 cellulose, which was purchased from Whatman International Ltd. (Maidstone, UK).

#### 11. Protein gel

Protein samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli, 1970. After electrophoresis, the proteins



were fixed with 10% trichloroacetic acid and stained with 0.05% Coomassie brilliant blue R-250 in destain. Excess dye was removed by incubating the gel in destain (30% methanol - 7% acetic acid). The gel was soaked in 40% ethanol before it was dried between two sheets of permeable cellophane.

## 12. Western blot and dot blot

For immunological characterization, proteins were electrophoretically transferred from a SDS-PAGE-gel onto a nitrocellulose membrane (pore diameter 0.45  $\mu$ m, Schleicher & Schuell, Dassel, Germany) with a dry blotting apparatus (Plexi-labo, Gent, Belgium). The filter was blocked for at least 2h in PBS pH 7.4 (14.5 mM phosphate buffer pH 7.4 - 150 mM NaCl) with 2.5% skim milk powder and 0.1 % Triton X-100 (blocking buffer). Incubation with the primary antibody, diluted in blocking buffer, was carried out at room temperature for 30 to 60 min. Excess of unbound antibody was removed by three washings with blocking buffer. The bound antibodies were detected with an alkaline phosphatase conjugated antibody of the appropriate specificity. Subsequently, the filter was washed two times with PBS pH 7.4 - 0.1% Triton X-100. A third washing step was carried out with substrate buffer (100 mM Tris-HCl pH 9.5 - 100 mM NaCl - 5 mM  $MgCl_2$ ). The filter was then incubated in substrate buffer with 165  $\mu$ g/ml nitro blue tetrazolium (NBT) and 165  $\mu$ g/ml 5-bromo-4-chloro-3-indolylphosphate (BCIP) until a clear signal appeared. The blot was finally washed thoroughly with tap water and dried.

The dot blot analysis was carried out in a similar way as the Western blot, except that the proteins were not transferred through electrophoresis, but by filtering the samples through a nitrocellulose membrane.

## 13. ELISA

In every ELISA a 0.1 % casein solution was used for blocking and for making the dilutions of the

antibodies used. The stock solution of casein (2.5%) was prepared as follows: 6.25 g casein powder was dissolved in 200 ml 300 mM NaOH by overnight stirring at 37°C. Then the pH was adjusted to 7.0 by adding 2N HCl. The final volume was brought to 250 ml (Nunc bulletin no. 7, December 1989). Sodium azide (0.02%) was added as a preservative.

Different ELISA's were developed to determine the concentration of Hepatitis B core or human  $\beta$ 2-microglobulin fusion proteins. Microtiter plates (type II F96 maxisorp Nunc A/S, Roskilde, Denmark) were coated for 1.5 h at room temperature or overnight at 4°C with a 1/2 dilution series of samples containing IPM2HBcm or IPM2hB2Mm. On the same plate, a 1/2 dilution series of purified HBc or hB2M, respectively, starting from 2  $\mu$ g/ml, was used as a standard. Between every incubation step, the plates were washed twice with tap water and once with PBS, pH 7.4 - 0.05% Triton X-100, except that after blocking, the plates were not washed. The microtiter plates were blocked with 0.1% casein solution for 2h at room temperature or at 4°C overnight. As primary antibody we used mouse anti-HBc or mouse anti-hB2M, respectively. The bound antibodies were detected with an alkaline phosphatase labelled anti-mouse IgG ( $\gamma$  chain specific) antibody. The incubation with antibody solution was carried out at room temperature for 1.5 h. Finally the microtiter plates were incubated for 1 h with substrate buffer (10% diethanolamine - 0.5 mM  $MgCl_2$  - 0.02%  $NaN_3$ , pH 9.8) containing 1 mg/ml p-nitrophenyl phosphate. The absorbance was measured at 405 nm and the wave length of 490 nm was used for normalization.

#### 14. Preparation of polyclonal anti-M2

All mice, which had been immunized with IPM2HBcm and had survived the lethal challenge with m.a. A/PR/8/34 influenza A virus (see results, immunization) were anaesthetized with 250  $\mu$ l 25 mg/ml tribromoethanol (injected i.p.) and blood samples were taken by heart

puncture. The serum was isolated as described hereinbelow. The crude serum gave a high background in Western blot, therefore an IgG fraction was prepared. The crude serum was filtered through a 0.45  $\mu$ m filter  
5 (Millipore Millex-HV, Millipore, Bedford, MA, USA) and diluted 10 times in loading buffer (PBS - 10 mM EDTA, pH 8). This mixture was loaded on an equilibrated Protein G Sepharose 4 Fast Flow column ( $\phi$  = 1 cm, h = 8 cm), The bound IgG molecules were eluted with 100 mM glycine-HCl,  
10 pH 2.7. Fractions of 1 ml were collected in tubes containing 50  $\mu$ l 1 M Tris-HCl pH 9.5 to bring the pH to neutral.

The quantity of anti-M2 antibodies in the pooled peak fractions was 2.6  $\mu$ g/ml. This was determined  
15 in an ELISA, comparable to the detection of anti-M2 antibodies in the serum of immunized mice. Mouse monoclonal anti-human  $\beta$ 2-microglobulin (Cymbus Bioscience, Southampton, UK) was used as a standard.

#### 20 15. Serum preparation

Five blood samples were taken from every mouse: the pre-immune serum (a), the serum taken after the first (b), after the second (c) and after the third (d) immunization, and the serum taken after challenge (e).  
25 This blood was incubated for 30 min at 37°C. The samples were then placed on ice for at least 1 hour and centrifuged two times 5 min at 16000 g in a microcentrifuge. The serum was isolated.

Equal volumes of sera obtained from different  
30 mice were pooled for the analysis of antibody production.

#### 16. RT-PCR

Allantoic fluid of A/Ann Arbor/6/60 (215 HAU) was incubated in AMV buffer (Boehringer, Mannheim,  
35 Germany) at 65°C for 30 min. 1/20 of this mixture was used for the reverse transcriptase (RT) reaction. Too this vRNA (genomic viral RNA) mixture 50  $\mu$ mol oligonucleotide (RT-NTRNA7), 10 mM DTT and 2.5 mM dNTP

was added. After an incubation of 10 min at 70°C, 20 units of AMV reverse transcriptase (Boehringer, Mannheim, Germany) and 40 units of RNase inhibitor (Boehringer, Mannheim, Germany) were added. The RT reaction was done at 42°C for 1 h. 1/3 of this reaction mixture was used for the PCR reaction as described earlier.

#### 17. Transfection and expression

HEKT cells were put in a 6 well plate at  $2 \times 10^5$  cells/well and grown for 24 h. 2 µg pDNA with FuGene TM 6 Transfection reagent (Boehringer, Mannheim, Germany) was added to the cells. 48 h after transfection the cells were lysed in 100 µl PBS, pH 7.4 - 5 mM EDTA - 0.5% Nonidet P40. The soluble fraction was isolated after 5 min centrifugation at 10,000 g. The pellet was resuspended in 100 µl PBS, pH 7.4.

#### 18. DNA vaccination

Plasmid DNA was used at a concentration of 1 µg/µl. Three intramuscular injections were given at three weeks intervals. Serum was taken two weeks after every immunization, pooled and analyzed in an ELISA for antibody response towards the extracellular part of the M2 protein (see Materials and Methods hereinabove).

#### 19. ELISA II

Microtiterplates were coated with 1 µg/ml M2, expressed in Sf9 insect cells (Black et al., 1993a, b). The remainder of the procedure was as described in the earlier section of Materials and Methods.

#### 20. List of plasmids

##### 20.1 E. coli

pATIPM2m1 : plasmid that contains the uninterrupted m2 gene from A/PR/8/34

pIPM2hB2Mm2s2 : plasmid for the expression of IPM2hB2Mm, with the correct amino terminus of M2

**pPLcIPM2HBc** : expression plasmid for IPM2HBc, with four amino acids between the initiating methionine and the amino terminus of M2e

5 **pPLcIPM2HBcm** : expression plasmid for IPM2HBcm, with the correct amino terminus of M2e. Sequence of M2 is derived from A/PR/8/34

**pPLcIM2HBcm** : expression plasmid for IM2HBcm, with the correct amino terminus of the universal M2

## 10 20.2 L. lactis

**pT1TT** : plasmid for the expression of TTFC

**pT1PM2LTT** : expression of IPM2TT, with leucine codons adapted for L. lactis. Sequence of M2e is derived from A/PR/8/34

15 **pT1PM2LTTIL2** : expression of IPM2TT, with adapted leucine codons, in combination with mIL2

**pT1PM2LTTIL6** : plasmid for the expression of IPM2TT, with adapted leucine codons, in combination with mIL6

20 **pT1HBc** : plasmid for the expression of HBc

**pT1HBcIL2** : expression of HBc in combination with mIL2

**pT1HBcIL6** : expression of HBc in combination with mIL6

25 **pT1PM2HBc** : plasmid for the expression of IPM2HBcm. Sequence of M2e is derived from A/PR/8/34

**pT1PM2HBcIL2** : expression of IPM2HBcm in combination with mIL2

30 **pT1PM2HBcIL6** : expression of IPM2HBcm in combination with mIL6

**pT1M2HBc** : plasmid for the expression of IM2HBcm, with the universal sequence for M2e

**pT1M2HBcIL2** : expression of IM2HBcm in combination with mIL2

35 **pT1M2HBcIL6** : expression of IM2HBcm in combination with mIL6

**pT1PM2LHBc** : plasmid for the expression of IPM2HBcm, with leucine codons adapted for L. lactis

- pT1PM2LHBcIL2 : expression of IPM2HBcm, with adapted  
 leucine codons, in combination with mIL2  
 pT1PM2LHBcIL6 : plasmid for the expression of  
 IPM2HBc, with adapted leucine codons, in combination  
 5 with mIL6  
 pT1M2LHBc : expression of IM2HBcm, with leucine  
 codons adapted for L. lactis  
 pT1M2LHBcIL2 : expression of IM2HBcm, with adapted  
 leucine codons, in combination with mIL2  
 10 pT1M2LHBcIL6 : expression of IM2HBcm, with adapted  
 leucine codons, in combination with mIL6  
 pT1cM2L : plasmid for the expression of the  
 cytoplasmic form of M2e, with leucine codons adapted  
 for L. lactis.  
 15 pT1cM2LC3d : expression of cM2LC3d, with adapted  
 leucine codons  
 pT1cM2LC3d3 : expression of cM2LC3d3 (with three  
 consecutive C3d domains), with adapted leucine  
 codons  
 20 pT1sM2LX : plasmid for the expression of the  
 secreted and anchored form of M2e, with leucine  
 codons adapted for L. lactis  
 pT1sM2LC3d : expression of sM2LC3d, with adapted  
 leucine codons  
 25 pT1sM2LC3d3 : expression of sM2LC3d3 (with three  
 consecutive C3d domains), with adapted leucine  
 codons
- 20.3  
 30 pUCM2 : plasmid that contains the  
 uninterrupted m2 gene from A/Ann Arbor/6/60  
 pCDNA3 : basic vector for eukaryotic gene  
 expression  
 pCIM2 : plasmid used for DNA vaccinations, it  
 35 carries the uninterrupted m2 gene from A/Ann  
 Arbor/6/60  
 pCIM2HBcm : plasmid used for DNA vaccinations, it  
 carries im2hbcm

001020-94086400

pCIP3M2HBcm: plasmid used for DNA vaccinations, it contains three times the extracellular domain of the M2 protein genetically fused to the Hepatitis B core protein. The fusion protein, IP3M2HBcm starts with the correct amino terminus of M2e. Sequence of M2 is derived from A/PR/8/34.

## EXPERIMENTAL SECTION

### 1. Construction of pATIPM2m

The RNA segment 7 of the influenza A virus, A/PR/8/34 (H1N1), was cloned by a procedure as described for RNA segment 4 in Min Jou et al., 1980. The resulting plasmid was named pATIPMA and is commercially available (LMBP catalogue 1992, no. 1774).

The mRNA of the M2 protein is not a collinear transcript of RNA segment 7. Indeed, an intron of 689 nucleotides had to be removed (Lamb et al., 1981).

In the plasmid pATIPMA, StuI cuts after the first nucleotide of the second exon (see figure 1a). This nucleotide was included in the synthetic oligonucleotides, that were used to code for the first exon. The synthetic first exon, encoding the amino-terminus of the mature M2 protein, was designed to contain a single stranded GATC overhang at its 5' end. This allowed us to make the connection to a preceding BamHI site in the vector pATIPMA and to replace the original first exon.

Furthermore codon usage was optimized for expression in E. coli.

Next, we introduced, by site-directed mutagenesis (Stanssens et al., 1989), a BclI site at the junction between the extracellular part and the membrane anchoring region of the M2 protein (see figure 1 b). The amino acid sequence of the extracellular part was not changed. The resulting plasmid, pATIPM2m1, carries the uninterrupted m2 gene of A/PR/8/34.

## 2. Construction of IPM2hB2Mm

Parker and Wiley (1989) expressed human  $\beta$ 2-microglobulin in the periplasm of *E. coli* by making use of the plasmid p714. This plasmid contains the coding region for  $\beta$ 2-microglobulin preceded by the signal sequence of the outer membrane protein A of *E. coli* (OmpA-ss) (see figure 2a). The OmpA signal sequence is required for the translocation of the protein, to which this sequence is fused, to the periplasm. The signal sequence is cleaved off after transport. On plasmid p714, human  $\beta$ 2-microglobulin is under control of both the lipoprotein (lpp) and lacUV5 promoter. Addition of 1 mM IPTG to a mid-log phase culture leads to the production of  $\beta$ 2-microglobulin.

The coding sequence of the extracellular part of the M2 protein, isolated as a BamHI-BclI fragment from pATIPM2m1, was inserted between the signal sequence of ompA and the human  $\beta$ 2-microglobulin (for details see figure 2a). Due to the construction, there were 9 additional nucleotides between the end of the ompa signal sequence and the beginning of the m2 fragment, which had to be removed (see figure 2b). This was done by looping out mutagenesis according to Nakamaye and Eckstein, 1986. As a result, the plasmid pIPM2hB2Mm2s2 was obtained.

## 3. Localization of the IPM2hB2Mm

A freshly grown preculture of C3000 containing p714 or pIPM2hB2Mm2s2 was diluted 1/100 in LB with ampicillin. As described above, the hb2m and ipm2hb2mm genes are under control of the lacUV5 promoter. When the cultures reached a density of about  $5.5 \times 10^8$  bacteria/ml, they were divided in two and one half of each culture was induced with 1 mM IPTG. After 3 h induction, the bacteria were harvested and fractionated. The periplasm of the bacteria was isolated by osmotic shock (Neu and Heppel, 1965). The remainder of the bacteria was sonicated (Vibra cell, Sonics & Materials Inc., Danbury, Conn., USA) and centrifuged for 10 min at 16000 g, to isolate the



cytoplasm. The different samples were analyzed on a SDS 15% PAGE-gel. Human B2M and the fusion protein IPM2hB2Mm were transported to the periplasm, whereas the precursors, still containing the signal sequence, remained associated with the bacteria. Determination of the amino-terminus of the mature IPM2hB2Mm (by courtesy of Dr. J. Vandekerckhove) by automated Edman degradation on a model 470A gas-phase sequencer coupled to a model 120A on-line phenylthiohydantoin amino acid analyzer (Applied Biosystems, Foster City, CA., USA), demonstrated that the OmpA signal sequence was correctly cleaved off.

#### 4. Purification of IPM2hB2Mm

The fusion protein IPM2hB2Mm could be expressed efficiently in the periplasm of *E.coli*. Whereas performing an osmotic shock is a critical procedure, especially on large volumes, Steidler et al. (1994) previously described an elegant system, based on the controlled expression of the Kil protein, to release periplasmic proteins in the growth medium.

The *kil* gene is present on a compatible plasmid under the tightly regulated  $P_L$  promoter, the leftward promoter of phage  $\lambda$  (Remaut et al, 1981). The plasmid pcI857K1 also carries the temperature sensitive repressor of the  $P_L$  promoter, cI857. The fusion protein IPM2hB2Mm is synthesized upon induction with 1 mM IPTG and at the end of the production phase, the culture is switched from 28°C to 42°C to induce Kil.

A fermentation (BioFlo IV fermentor, New Brunswick Scientific Co., Edison, N.J., USA) was carried out using the standard induction procedure described above. The culture was centrifuged in a centrifuge 17RS (Heraeus Instruments, Hanau, Germany) at 11000 g and the growth medium was isolated. The sodium chloride concentration of the growth medium was adjusted to 300 mM and buffered with 20 mM MES (2-(N-morpholino)ethanesulphonic acid), pH 6.5. This solution was loaded on a DEAE Sephacel column ( $\phi$  = 5 cm, h = 6.5

cm), equilibrated with 20 mM MES, pH 6.5 - 300 mM NaCl. Under these conditions IPM2hB2Mm did not bind to the matrix. The ammonium sulphate concentration of the flow through was brought to 0.8 M with a 3.8 M  $(\text{NH}_4)_2\text{SO}_4$  solution, pH 7. The mixture was loaded on a Phenyl Sepharose column ( $\phi$  = 5 cm, h = 17 cm), equilibrated in 20 mM Tris-HCl, pH 7.5, 0.8 M  $(\text{NH}_4)_2\text{SO}_4$ . A decreasing ammonium sulphate concentration gradient starting from 0.8 M and going to 0, did not release the bound fusion protein. This was achieved by eluting the column with a pH gradient from 20 mM Tris-HCl, pH 7.5 to 5 mM NaAc, pH 5.5. The peak fractions were pooled and diluted ten times in 20 mM diethylamine (DEA), pH 8.5.

The complete mixture was loaded on a Sepharose Q column ( $\phi$  = 0.8 cm, h = 2.3 cm), equilibrated with 20 mM DEA, pH 8.5. The protein was eluted from the column with a salt gradient from 0 to 1 M. The peak fractions were pooled and loaded on a Sephacryl S-100 gel filtration column ( $\phi$  = 1.5 cm, h 47 cm). Only one peak with the expected molecular weight of about 15 kDa was observed. This purified IPM2hB2Mm was used to immunize mice for preparing hybridomas, secreting monoclonal antibodies directed against the M2 protein.

#### 25 5. Production of monoclonal antibodies to the M2 protein

Balb/c mice were immunized three times with 2.5  $\mu\text{g}$  purified IPM2hB2Mm. For the first injection a complete dose of Ribi adjuvant was used. The second and third immunization were performed in the presence of 50  $\mu\text{g}$  MPLA. The injections were given with an interval of three weeks. Three days after the last immunization, spleen cells were isolated and fused with myeloma cells SP2/0-AG14 using standard protocols (Köhler and Milstein, 1975). Supernatants from different immunoglobulin producing cell clones were tested in ELISA and Western blot for reactivity against the other fusion protein IPM2HBcm (described further). The Hepatitis B core protein alone was used as a control to eliminate false

positive clones. The isotype of the antibody was determined (Isostrip, Boehringer, Mannheim, Germany). Two different immunoglobulin subtypes that recognized the extracellular part of the M2 protein were obtained, an IgM and an IgG2a. Especially the IgG2a antibody was used in further experiments.

#### 6. Expression of HBc and IPM2HBcm

Expression of proteins under control of the  $P_L$  promoter was performed by shifting an exponentially growing culture from 28°C to 42°C (Remaut et al., 1981). A saturated preculture of MC1061 [pcI857] containing the plasmid pPLc245 (control), pPLcA1 (carrying the *hbc* gene) or pPLcIPM2HBcm (containing the fusion gene *ipm2hbc*) respectively, was diluted 1/100 in LB medium (50 µg/ml kanamycin and 100 µg/ml ampicillin) and grown for about 4 h at 28°C under shaking. When the cultures reached a density of  $4.5 \times 10^8$  to  $5.5 \times 10^8$  bacteria/ml, they were split, one half was incubated for 4 h at 28°C, the other half was switched to 42°C. The bacteria were concentrated by centrifugation (2 min at 16000 g in a microcentrifuge).

The culture medium was removed and the bacteria were resuspended in TE buffer (10 mM Tris-HCl - 1 mM EDTA, pH 7.6). The bacteria were opened by sonication (Vibra cell, Sonics & Materials Inc., Danbury, Conn., USA) and the bacterial debris were pelleted for 10 min at 16000 g in a microcentrifuge. The supernatant was isolated and the pellet was resuspended in TE buffer. The samples were analyzed on a SDS 12.5% PAGE-gel, in a Western blot and on a dot blot.

#### 7. Large scale production of IPM2HBcm

The strain MC1061 [pcI857, pPLcIPM2HBcm] was grown in a BioFlo IV fermentor (New Brunswick Scientific Co., Edison, N.J., USA). When the culture reached a density of about  $5.5 \times 10^8$  cells/ml, the temperature was increased to 42°C. After three hours of induction, the

culture was centrifuged in a centrifuge 17RS (Heraeus Instruments, Hanau, Germany) at 11,000 g. The bacteria were collected and resuspended in a volume (in ml) buffer (50 mM Tris-HCl pH 8 - 150 mM NaCl - 5% glycerol with one protease inhibitor cocktail tablet (Complete™; Boehringer, Mannheim, Germany) per 25 ml) corresponding to two times the weight (in g) of the pelleted bacteria. This suspension was treated with 1 mg/ml lysozyme (freshly dissolved in 25 mM Tris-HCl, pH 8) for half an hour on ice. Subsequently, the bacteria were lysed with 0.2% Triton X-100 in the presence of 25 mM EDTA, pH 8. After 30 min incubation on ice, the lysates were centrifuged for 1 h in a Sorvall SS-34 rotor (Du Pont Company, Wilmington, DE, USA) at 48000 g. The supernatant was removed and used for purification of IPM2HBcm.

#### 8. Immunization with IPM2HBcm

Balb/c mice were injected three times intraperitoneally with purified IPM2HBcm in the presence of adjuvant. Control mice received only PBS buffer, pH 7.4 and adjuvant. For the first immunization half a dose of Ribi adjuvant was used. In the second and third injection we used 25 µg MPLA and 25 µg MDP.

Mice were immunized intranasally three times by applying a light ether anaesthesia, after which 50 microliter antigen solution in PBS buffer (containing either 10 microgram IPM2HBcm or IM2HBcm without any adjuvant) is put in the nostril.

#### 9. Expression in *L. lactis*

Single colonies from *L. lactis* strain MG 1363, containing the plasmid pT1HBc, pT1PM2HBc or pT1M2HBc, respectively, or the derivatives with mIL2 (pT1HBcIL2, pT1PM2HBcIL2 and pT1M2HBcIL2) or mIL6 (pT1HBcIL6, pT1PM2HBcIL6 and pT1M2HBcIL6), were inoculated in 10 ml GM17E each. MG1363 [pTREX1] was used as control. The bacteria were grown for about 16 h at 28°C. The cells were collected by centrifugation at 2000 g for 20 min

(Sorvall 11 RT6000 D). The growth medium was isolated and the bacteria were resuspended in 250  $\mu$ l TE. Following resuspension, an additional 250  $\mu$ l TE supplemented with 10 mg/ml lysozyme and 200 u/ml mutanolysin was added.

5 This mixture was incubated for 10 min at 37°C and then put on ice for 5 min. Then 500  $\mu$ l Laemmli sample buffer (100 mM Tris-HCl pH 6.8 - 5% SDS - 1.2M  $\beta$ -mercaptoethanol - 0.008% bromophenol blue - 16% glycerol) was added and the samples were boiled for 5 min. An equivalent of 1 ml

10 original culture volume, or  $10^9$  bacteria was analyzed on a SDS 12.5% PAGE-gel. The production of mIL2 or mIL6 in the culture supernatant was evaluated in a bio-assay based on the proliferation of CTLL2-cells (mIL2, Gillis et al., 1978) or the proliferation of a B-cell hybridoma, 7TD1

15 (mIL6, Van Snick et al., 1986).

#### 10. Passive immunization

The purified preparation of IM2HBcm particles was used to immunize 7 weeks old female Balb/c mice. A

20 total of 40 mice were immunized with 10 pg IM2HBcm. A control group of 40 mice only received buffer. A total of three injections combined with appropriate adjuvant were given at three weeks intervals (see Materials and Methods). Two weeks after the third immunization 28 mice

25 from each group were bled and serum was isolated (see Materials and Methods). This serum was administered intraperitoneally to naive mice 24 h before infection. This process is called passive immunization. Twelve mice received 800  $\mu$ l serum from IM2HBcm immunized mice and

30 another 12 mice received serum from the control group. These 24 mice and the remaining 24 immunized mice were challenged with 5 LD<sub>50</sub> m.a. X47 three weeks after the third immunization. The virus was administered intranasally in a total volume of 50  $\mu$ l after ether

35 anaesthesia. Morbidity was followed by measuring rectal temperature and weight every other day.

### 11. Constructs for DNA vaccination (Fig. 29)

The mammalian expression vector, pCDNA3 (Invitrogen, Leek, The Netherlands), which carries the cytomegalovirus promoter was used to make the different DNA vaccination vectors.

The uninterrupted m2 gene was isolated by RT-PCR from the influenza A virus A/Ann Arbor/6/60 (see Materials and Methods). The amplified fragment was cut with BglII and XbaI and inserted into the BglII and XbaI opened pUC19. This plasmid was called pUCM2. The sequence of the m2 gene was determined and shown to correspond to the cold adapted form of the gene. The m2 gene was isolated from pUCM2 as a 321 bp EcoRI-XbaI fragment and inserted into the EcoRI and XbaI opened pCDNA3. This resulted in plasmid pCIM2.

Two fusion genes, ip3m2hbcm and im2hbcm, were also inserted into pCDNA3. The im2hbcm gene was amplified by PCR from pPLcIM2HBcm. This fragment was cut with SpeI and phosphorylated with T4 polynucleotide kinase. This 630 bp fragment was inserted in the EcoRV and XbaI opened pCDNA3. The resulting plasmid was called pCIM2HBcm.

During the construction of pPLcIPM2HBc (see figure 3a) plasmids were also obtained with two and three inserted M2e fragments. These plasmids were called pPLcIP2M2HBc and pPLcIP3M2HBc, respectively. The ip3m2hbcm gene was amplified by PCR from pPLcIP3M2HBc. This fragment was cut with SpeI, phosphorylated with T4 polynucleotide kinase and inserted in the EcoRV and XbaI opened pCDNA3. This plasmid was called pCIP3M2HBcm.

Plasmid DNA was isolated with an EndoFree Plasmid Giga kit (Qiagen, Hilden, Germany). The concentration pDNA was determined by spectrophotometric analysis.

### 12. Expression in HEKT cells

The plasmids pCDNA3, pCIM2, pCIM2HBcm and pCIP3M2HBcm were transfected to HEKT cells (see Materials

and Methods). 48h after transfection the cells were lysed and analyzed in a Western blotting experiment.

### 13. Analysis of the serum

Two weeks after every immunization serum samples were taken and analyzed in an ELISA. In panel A from figure 31 the two vectors, which can express the HBc fusion proteins are compared with the control vector. The ELISA was performed as described in Materials and Method.

## **RESULTS**

### 1. Construction of IPM2HBcm

The plasmid pPLcA1 (see figure 3a) contains the hepatitis b core (hbc) gene under control of the P<sub>L</sub> promoter of bacteriophage λ (a gift from Dr. Nassal). The 346 bp NcoI-XbaI HBc fragment, isolated from pPLcA1, was inserted into the NcoI and XbaI opened pMa581, a derivative of pMa58. This plasmid was called pMaHBc. At the 5' end of the hepatitis B core, directly following the start codon, we introduced a BamHI site by site-directed mutagenesis (Stanssens et al., 1989), correctly positioned in the reading frame of HBc (for details see figure 3a and b). The resulting plasmid was named pMaHBcm. The information coding for the extracellular part of the M2 protein was cloned as a 72 bp BamHI-BclI fragment, derived from pATIPM2m1, into the BamHI opened pMaHBcm, resulting in the vector pIPM2HBc. The hbc gene in the expression vector pPLcA1 was then replaced by the 418 bp NcoI-XbaI m2hbc fragment, creating pPLcIPM2HBc. Due to the construction, four amino acids extra were present between the first methionine and the start of the extracellular part of the M2 protein and had to be removed (see figure 3c). This was done by looping out mutagenesis (Deng and Nickolov, 1992). The resulting plasmid was named pPLcIPM2HBcm (see figure 3a and c).

## 2. Expression of the fusion protein

The plasmids pPLc245 (control), pPLcA1 (hbc gene) and pPLcIPM2HBcm (ipm2hbc gene) were transformed to MC1061 [pcI857]. After culture and induction, the bacteria were lysed by sonication. The lysates were centrifuged and an aliquot of the supernatants was loaded on a SDS 12.5% PAGE-gel (see figure 4). The same fractions were also analyzed by a Western blot. Two different monoclonal antibodies were used : an antibody specific for the Hepatitis B core protein and a monoclonal antibody (IgG2a) directed against the extracellular part of the M2 protein.

The monoclonal antibody against Hepatitis B core revealed two different bands (see figure 5A), one corresponding to the Hepatitis B core protein and the other to the fusion protein. The latter protein has a lower mobility, corresponding to the insertion of the extracellular domain of the M2 protein. The presence of the M2 fragment was confirmed by using the antibody specific for the extracellular part of the M2 protein (see figure 5B).

The N-terminal amino acid sequence of IPM2HBcm was determined (Dr. J. Vandekerckhove) by automated Edman degradation on a model 470A gas-phase sequencer coupled to a model 120A on-line phenylthiohydantoin amino acid analyzer (Applied Biosystems, Foster City, CA., USA). This analysis revealed the N-terminal sequence Ser-Leu-Leu, which is exactly the same as the amino terminal sequence of the M2 protein of the influenza A virus (figure 6). The first amino acid, methionine, was removed in E. coli. The amino-terminus of the fusion protein thus corresponds to that of the wild type M2 protein (table 1; Lamb et al., 1985).

Hepatitis B core, also when expressed in E. coli, spontaneously associates to form particles, indistinguishable from the viral core particles circulating in the blood of Hepatitis B infected patients (Cohen and Richmond, 1982). Clarke and co-workers (1987)



showed that a peptide inserted at the amino terminus of the Hepatitis B core protein could be detected at the surface of the particle.

Electron micrographs (Dr. G. Engler) showed that the IPM2HBcm fusion protein was able to form similar particles. To investigate whether the insertion of the extracellular part of the M2 protein resulted in the surface localization of this fragment, soluble fractions, containing HBC or IPM2HBcm, were loaded on a nitrocellulose membrane in a dot blot. The dot blots were treated with a monoclonal antibody directed against HBC or against M2. Figure 7 clearly shows a signal in the soluble pPLcIPM2HBcm fraction, when revealed with the antibody directed against the M2 protein (panel B). Since the soluble fraction is loaded in a native state onto the nitrocellulose membrane, we conclude that the epitope is located at the surface of the Hepatitis B core particle.

### 3. Purification of IPM2HBcm

The bacterial lysates were prepared as described in Materials and Methods. The concentration of Tris-HCl, pH 8 and NaCl were adjusted to 20 mM and 50 mM respectively. This mixture was loaded on a DEAE Sepharose column ( $\phi = 2.5$  cm,  $h = 5.5$  cm), equilibrated with 20 mM Tris-HCl, pH 8-50 mM NaCl. The fusion protein was not retained on the column. To the flow through 3.8 M  $(\text{NH}_4)_2\text{SO}_4$ , pH 7, was added to a final concentration of 1.2 M. This mixture was incubated under stirring in the cold room during 16h. The precipitate was removed over a CF11 cellulose column ( $\phi = 2.5$  cm,  $h = 3.5$  cm). The column was eluted with PBS, pH 7.4. The eluate of about 50 ml was concentrated in a Centiprep 30 (Amicon Corporation, Danvers, Ill., USA) to 5 ml and loaded on a Sephacryl S-300 column ( $\phi = 2.5$  cm,  $h = 91$  cm), which was equilibrated with PBS, pH 7.4. The peak fractions were pooled and the concentration of IPM2HBcm was determined in an ELISA, The LPS content was assayed (LAL Coatest® Endotoxin purchased from Endosafe Inc., Charleston, SC.,

#### 4. Immunization

All mice immunized with IPM2HBcm showed a significant degree of protection against the following influenza challenge. Depending on the administered dose, 9 to 11 mice out of 12 survived the influenza infection, versus only 2 out of 11 for the control group (see figure 8B).

One day prior to the first (bleeding a) and two weeks after every injection (bleeding b, c and d) blood samples were taken. Three weeks after the challenge, when the mice had recovered sufficiently from the influenza infection, a last blood sample (e) was taken. The serum was analyzed in an ELISA (see Materials and methods) to identify IgG antibodies directed towards the extracellular part of the M2 protein. To do so, we made use of the other fusion protein, IPM2hB2Mm. One half of the microtiter plate was coated with human  $\beta$ 2-microglobulin, the other half was coated with the fusion protein IPM2hB2Mm, both as unpurified culture supernatant. The concentration of IPM2hB2Mm used was 1

µg/ml. The same concentration of total protein was used in both set ups. Therefore, the hB2M content of the culture supernatant of bacteria expressing hB2M had to be adjusted to 1 µg/ml by adding purified hB2M (Sigma Chemical Co., St. Louis, Mo., USA). Dilution series (1/3) of the different serum samples, starting from 1/50, were loaded on the hB2M and IPM2hB2Mm, coated wells. The ELISA was further developed as described in Materials and methods.

10 To obtain the value for the specific reactivity towards the extracellular part of the M2 protein, the absorbance of hB2M at a given dilution was subtracted from the absorbance of IPM2hB2Mm of the corresponding dilution. Figure 9 clearly demonstrates a high antibody  
15 response to the extracellular part of the M2 protein, in the mice which received three injections with the vaccine. The titer in the serum was further increased after the challenge.

## 20 6. Construction of IM2HBcm

It is the aim of the present invention to make a universal vaccine against influenza A viruses. In the vaccination studies described above, we showed protection against the influenza virus from which the original M2  
25 sequence was derived, A/PR/8/34 (homologous protection). The extracellular part of the M2 protein from this virus differs from most other viruses sequenced to date, by only one amino acid (see table 1). Therefore, a construct was made in which the glycine at position 20 was changed  
30 to aspartic acid.

To do so we made use of an intermediate vector in the construction of pPLcIPM2HBcm, pMaIPM2HBc2 (see figure 3a). The plasmid pMaIPM2HBc2 does not yet contain the mutated m2 (deletion of 12 extra nucleotides)  
35 fragment, which starts at the first mature codon of the M2 protein. Therefore this fragment was isolated from pPLcIPM2HBcm by cutting with SgrAI and EcoRI. This 499 bp SgrAI-EcoRI fragment was cloned into the SgrAI and EcoRI

opened vector pMaIPM2HBc2, which resulted in the construction of pMaIPM2HBc3 (see figure 10).

By site-directed mutagenesis according to Deng and Nickoloff (1992) the sequence of the extracellular part of the M2 protein was changed to the more universal M2 sequence (Gly20 -> Asp). The new plasmid was called pIM2HBcm. The sequence was determined on a model 373A sequencer (Applied Biosystems, Foster city, CA., USA) and shown to contain the desired mutation. The mutated M2 fragment was isolated from pIM2HBcm as a 499 bp SgrAI-EcoRI fragment and reintroduced into the expression vector pPLcIPM2HBcm, opened with SgrAI and EcoRI, to create pPLcIM2HBcm.

#### 7. Expression of IM2HBcm

Strain MC1061 [pcI857] containing respectively pPLc245, pPLcA1, pPLcIPM2HBcm or pPLcIM2HBcm was cultured as described in the Experimental Section. The bacteria were collected and opened by sonication. The soluble fraction was isolated and the concentration of Hepatitis B core protein or the derived fusion proteins was determined in an ELISA. A soluble fraction containing 5 µg HBc or I(P)M2HBcm was analyzed on a SDS 12.5% PAGE-gel (see figure 11). The same fractions were also analyzed in a Western blot (see figure 12). The proteins of interest were detected with an antibody directed against the Hepatitis B core protein or with the monoclonal antibody specific for the extracellular part of the M2 protein. It can be concluded that the new fusion protein, IM2HBcm, is expressed as efficiently as IPM2HBcm. Moreover the amino acid change in the extracellular part of the M2 protein (Gly20 --> Asp) has no effect on the binding of the monoclonal anti-M2 antibody.

#### 8. Immunization against heterologous challenge

A similar procedure as described in point 4 was used to test the efficiency of IPM2HBcm and IM2HBcm to protect mice versus heterologous challenge with

influenza. 10 microgram of IPM2HBcm or IM2HBcm (purified in an identical way as IPM2HBcm) was used for immunization. The mice were challenged with 30 HAU X-47.

All mice immunized showed a significant degree of protection against the heterologous challenge. 8 (in case of IPM2HBcm,  $p < 0.05$ ) or 12 (in case of IM2HBcm,  $p < 0.0001$ ) mice out of 12 survived the influenza infection, versus only 2 out of 11 in the control group (figure 8C).

To test the effect of intranasal administration, the same procedure was followed, but instead of the intraperitoneal injection, the antigen was administered intranasally. Also in this case, the protection is evident: 12 (in case of IPM2HBcm,  $p < 0.0001$ ) or 11 (in case of IM2HBcm,  $p < 0.001$ ) mice out of 12 survived the influenza infection, versus 2 out of 11 in the control group (figure 8D).

#### 9. Construction of vectors for the expression of M2-HBc fusion proteins in *L. lactis*

The plasmid pTREX1 (Wells and Schofield, 1996) was used to express the Hepatitis B core protein and two M2-HBc fusion proteins, IPM2HBcm and IM2HBcm, in *Lactococcus lactis*. This plasmid has a constitutive *L. lactis* chromosomal promoter, P<sub>l</sub>, which is followed by the translation initiation region of the *E. coli* bacteriophage T7 gene 10 (Wells and Schofield, 1996). The transcription terminator is derived from T7 RNA polymerase. The plasmid pTREX1 also carries two genes for resistance to erythromycin.

The expression plasmid, pTREX1, was cut with SphI, leaving a 3'CATG extension which was removed with Klenow DNA polymerase. The removed nucleotides were included in the sense linker for PCR amplification of the different genes. The linearized vector was then cut with BamHI and treated with CIP (calf intestine phosphatase, Boehringer, Mannheim, Germany).

The genes hbc, ipm2hbc and im2hbc were amplified by PCR (see Materials and methods). The anti-sense linker (HBca) was identical in all amplifications and provided a SpeI and a BclI site after the stop codon 5 (see figure 13). For the amplification of ipm2hbc and im2hbc the same sense oligonucleotide (M2s) could be used, since the mutation Gly → Asp in the extracellular part of the M2 protein is located further downstream.

The amplification of hbc from pPLcA1 was only possible after the vector had been linearized with ScaI. The amplification reaction that produced a sufficient amount of fragment, under the most stringent conditions, was used for further cloning. The amplified fragment, hbc, ipm2hbc or im2hbc, was cut with BclI, phosphorylated 15 with T4 polynucleotide kinase and inserted in the SphI and BamHI opened pTREX1 (see figure 14). The new plasmids were called pT1HBc, pT1PM2HBc (in which the extracellular part of the M2 protein is derived from the virus A/PR/8/34) and pT1M2HBc (in which the sequence of the 20 extracellular part of the M2 protein corresponds to the type present in nearly all human influenza A viruses sequenced to date), respectively. The sequence of the inserted fragment was determined on a model 373A sequencer (Applied Biosystems, Foster City, CA., USA) and 25 shown to be correct.

In view of using Lactococcus lactis as an improved vaccine delivery vehicle, two murine cytokines, interleukin 2 (mIL2) and interleukin 6 (mIL6) were inserted as second cistrons in the same operon as the 30 antigen. In that way we could obtain bacteria expressing the antigen, e.g. IM2HBcm, together with secreted murine interleukin 2 or 6. To obtain secretion of the interleukins into the growth medium, they were fused in frame to the lactococcal usp45 secretion signal peptide 35 (van Asseldonk et al., 1990). The plasmids pT1HBc, pT1PM2HBc and pT1M2HBc were cut with SpeI and treated with CIP. The murine interleukin 2 gene was isolated as a 572 bp XbaI-SpeI fragment from plasmid pL2MIL2 (Steidler

et al., 1995). This fragment was inserted into the SpeI opened pT1HBc, pT1PM2HBc and pT1M2HBc giving rise to pT1HBcIL2, pT1PM2HBcIL2 and pT1M2HBcIL2, respectively. In an analogous way the murine interleukin 6 gene was isolated as a 687 bp XbaI-SpeI fragment from pL2MIL6 (Steidler et al., 1996) and inserted into the SpeI opened vectors, pT1HBc, pT1PM2HBc and pT1M2HBc, to create pT1HBcIL6, pT1PM2HBcIL6 and pT1M2HBcIL6, respectively.

10 10. Expression of HBc and M2HBc in L. lactis

Lactococcus lactis strain MG1363 (Gasson, 1983) containing the plasmids for the expression of the antigen alone (pT1HBc, pT1PM2HBc and pT1M2HBc) or in combination with mouse interleukin 2 (pT1HBcIL2, pT1PM2HBcIL2 and pT1M2HBcIL2) or mouse interleukin 6 (pT1HBcIL6, pT1PM2HBcIL6 and pT1M2HBcIL6) were cultured as described in Materials and Methods. MG1363 [pTREX1] was used as control.

An equivalent of  $10^9$  bacteria was analyzed by SDS 12.5% PAGE. The expression of the Hepatitis B core and the M2-HBc fusion proteins were analyzed by Western immunoblotting (see figure 15) carried out as described in Materials and methods. The expression of IM2HBc in MG1363 [pT1M2HBcIL6] was not as high as in the other constructs. By screening different colonies a clone could be isolated with comparable expression levels.

The production and secretion of interleukins into the growth medium was analyzed in a biological assay. The biological activity of mIL2 was assayed by the proliferation of a T-cell line, CTLL2 (Gillis et al., 1978) as compared to a human IL2 standard. The biological activity of mIL6 was measured by the proliferation of a B-cell hybridoma, 7TD1 (Van Snick et al., 1986). Table 2 gives an overview of the level of interleukin 2 and 6 per ml culture medium produced by the different expression plasmids. The supernatant of cultures producing mIL6 did not lead to proliferation in a mIL2 assay and vice versa.

Table 2

Plasmid	mIL2 production	mIL6 production
pT1HBcIL2	410 ng/ml	-
pT1PM2HBcIL2	481 ng/ml	-
5 pT1M2HBcIL2	359 ng/ml	-
pT1HBcIL6	-	1020 ng/ml
pT1PM2HBcIL6	-	772 ng/ml
pT1M2HBcIL6	-	802 ng/ml

# 10 11. Adaptation of the coding sequence of M2e to expression in *L. lactis*

Since the two fusion proteins, IPM2HBcm and IM2HBcm could hardly be detected in a Western blot, we proceeded to augment the production of these two fusion  
 15 proteins by adapting the codon usage of the extracellular part of the M2 protein to *L. lactis* (van de Guchte et al., 1992).

At the 5' end of the extracellular part of the M2 protein we observed two consecutive leucine codons  
 20 (CUG CUG) that were optimal for expression in *E. coli* (68%), but poor for translation in *L. lactis* (8%, percentages described in van de Guchte et al., 1992). Therefore these codons were changed to UUA. The genes for ipm2hbc and im2hbc were amplified by PCR from  
 25 respectively pPLcIPM2HBcm or pPLcIM2HBcm, with a new sense primer, M2Ls, containing the two changed leucine codons (see figure 13). As anti-sense primer we used again HBca (see figure 13). The cloning of the genes was analogous as depicted in figure 14. The vectors so  
 30 created were called pT1PM2LHBC and pT1M2LHBC.

The expression level of the mutated M2HBc proteins, compared to the original fusion proteins, was analyzed in a Western blot (see figure 16). The expression level of the M2HBc fusion proteins with the *L.*  
 35 *lactis* adapted leucine codons, was indeed much higher. It



is concluded that the adaptation of codon usage to the L. lactis translation machinery, has a positive effect on the level of protein produced. In a similar way as described above, the murine interleukin 6 gene was inserted into pT1PM2LHBc and pT1M2LHBc, giving rise to pT1PM2LHBcIL6 and pT1M2LHBcIL6, respectively.

## 12. Construction of M2C3d in Lactococcus lactis

A second carrier protein, C3d, is also an attractive molecule for the presentation of the extracellular part of the M2 protein. Dempsey et al. (1996) demonstrated that the attachment of an antigen to three consecutive C3d molecules, was much more efficient in producing a high antibody response than the antigen administered in complete Freund's adjuvant.

The universal sequence of the extracellular part of the M2 protein, with the adapted leucine codons, was used for making a fusion to the amino-terminus of the first C3d molecule. The coding sequence for three different fusion proteins were constructed. In the first example the M2C3d3 fusion protein is expressed in the cytoplasm of L. lactis (cM2C3d3), similar to the M2HBc fusion proteins. In the second case the M2C3d3 protein is secreted into the growth medium by making an in frame fusion to the usp45-signal sequence (sM2C3d3), and the last construct, which is a derivative of the secreted form, contains in addition an anchor sequence (spaX) after the last C3d molecule to attach the fusion protein covalently in the cell wall (sM2C3d3X).

The amplified C3d3 fragment was first subcloned in a derivative of pUC18, namely pUCB/S. pUC18 was linearized with HindII and a BglII linker was inserted. The resulting plasmid was then opened with SmaI and a SpeI linker was inserted, resulting in the plasmid pUCB/S (see figure 18). Three succeeding copies of C3d were amplified from pSG5.C3d3.YL (a gift from Dr. D. Fearon) by PCR with the oligonucleotides C3ds and C3da (see figure 17). This amplified fragment was cut with BglII

and SpeI. The resulting 2830 bp BglIII-SpeI fragment was cloned into the BglIII and SpeI opened vector pUCB/S (see figure 18). The genes cm2 and sm2 were amplified by PCR. For the amplification of cm2 we used the sense  
5 oligonucleotide M2Ls (see figure 13) and the anti-sense linker M2Ca, which carried for our purposes a BamHI site in the correct reading frame (see figure 17). The same anti-sense linker was used for the amplification of sm2. The sense oligonucleotide for the amplification of sm2,  
10 M2LSs, started at the first codon of the mature M2 protein.

For the synthesis of the cytoplasmic form of M2C3d3, the information coding for the extracellular part of the M2 protein was inserted into pTREX1 analogous as  
15 the m2hbc gene described above (see also figure 18). The amplified cm2 fragment was cut with BamHI (77 bp), phosphorylated with T4 polynucleotide kinase and inserted in the SphI and BamHI opened pTREX1, creating pT1cM2L. For the synthesis of the secreted and anchored form of  
20 M2C3d3, the information coding for the extracellular part of the M2 protein was inserted into pT1NX. The vector pT1NX carries the usp45-signal sequence (usp45-ss) and the anchor sequence derived from Staphylococcus aureus protein A (spaX). The plasmid pT1NX was cut with NaeI,  
25 correctly positioned at the end of the usp45-ss and BamHI. The amplified fragment, sm2, was cut with BamHI and phosphorylated with T4 polynucleotide kinase. This 73 bp sm2 fragment was inserted into the NaeI and BamHI opened pT1NX, resulting in the plasmid pT1sM2LX (see  
30 figure 18). One single C3d fragment, isolated from pUCC3d, can then be inserted into the BamHI site at the end of the cm2 or sm2 sequence. Afterwards one or two additional C3d copies can be inserted.

### 35 13. Construction of M2TTFC in Lactococcus lactis

A third carrier protein, tetanus toxin fragment C (TTFC), can also be used. TTFC has already been expressed in L. lactis under control of the P1 promoter,

pT1TT (Wells and Schofield, 1996). L. lactis expressing TTFC in combination with mIL2 or mIL6 to raise the antibody production, was successfully used in immunization experiments (Patent GB 9521568.7).

5 As positive control for analysis of antibody response in the present immunization experiments with L. lactis expressing I(P)M2HBcm, a fusion was made between the extracellular part of the M2 protein and the amino terminus of TTFC.

10 The ttfc gene was amplified by PCR (see Materials and methods) from pT1TT. The sense oligonucleotide (TTFCs) provided a BamHI site, positioned in the correct reading frame, before the second codon of ttfc, corresponding to threonine. The anti-sense linker  
15 (TTFCa) provided a SpeI and a BamHI site after the stop codon (see figure 19). The amplification reaction that produced a sufficient amount of fragment, under the most stringent conditions, was used for further cloning (see Materials and methods). The amplified ttfc fragment was  
20 cut with BamHI, phosphorylated with T4 polynucleotide kinase and inserted in the BclI opened pATIPM2m1 (see figure 20). This plasmid construct was called pATIPM2TT. From this plasmid the m2ttfc gene was amplified by PCR (see Materials and methods) with M2Ls and TTFCa (see  
25 figure 19). The amplified m2ttfc fragment was cut with BamHI, phosphorylated with T4 polynucleotide kinase and inserted in the SphI and BamHI opened pTREX1 (see figure 20). The new plasmid was called, pT1PM2LTT. In this construct the extracellular part of the M2 protein is  
30 derived from the virus A/PR/8/34, with the two leucine codons adapted for use in L. lactis. The sequence of the inserted fragment was determined on a model 373A sequencer (Applied Biosystems, Foster City, CA., USA) and shown to be correct.

35 The murine interleukin genes, mIL2 and mIL6, were inserted in the same operon as m2ttfc. The murine interleukin 2 gene was isolated as a 572 bp XbaI-SpeI fragment from plasmid pL2MIL2 (Steidler et al., 1995).

This fragment was inserted into the SpeI opened pT1PM2LTT giving rise to pT1PM2LTTIL2 (see figure 20). In an analogous way the murine interleukin 6 gene was isolated as a 687 bp XbaI-SpeI fragment from pL2MIL6 (Steidler et al., 1996) and inserted into the SpeI opened vector pT1PM2LTT to create pT1PM2LTTIL6 (see figure 20).

#### 14. Expression of TTFC and M2TTFC in *L. lactis*

*Lactococcus lactis* strain MG1363 (Gasson, 1983) containing the plasmids for the expression of the antigen alone (pT1PM2LTT) or in combination with mouse interleukin 2 (pT1PM2LTTIL2) or mouse interleukin 6 (pT1PM2LTTIL6) were cultured as described in Materials and Methods. MG1363 [pT1TT] was used as a control. An equivalent of  $10^9$  bacteria was analyzed by SDS 10% PAGE. The expression of the IPM2TTFC fusion protein was analyzed by Western immunoblotting (see figure 21) carried out as described in Materials and Methods. The production and secretion of interleukins into the growth medium was analyzed by a biological assay. *L. lactis* [pT1PM2LTTIL2] produced about 500 ng/ml mIL2 and *L. lactis* [pT1PM2LTTIL6] about 1  $\mu$ g/ml mIL6. These results are comparable with the expression levels obtained with I(P)M2HBcm in combination with the two interleukins.

#### 15. Construction of pACsgpM2C3d3 and generation of the corresponding recombinant baculovirus

The amplified sequence of the baculovirus gp67 secretion signal was cut with SpeI and HindIII, and then subcloned in the SpeI-HindIII vector fragment of pUCC3d, resulting in pUCsgp. After HindIII and NaeI digestion of pUCsgp, the gp67 secretion signal was ligated with a HindIII treated M2e fragment (universal sequence) obtained from a PCR amplification (primers M2Ss and UM2ECa). This construct, referred to as pUCsgpM2, was digested with BamHI and subsequently recirculized by

ligation with the BglII-BamHI pUCC3d3 fragment containing 3 consecutive C3d fragments, yielding pUCsgpM2C3d3.

The latter fragment was excised after ligation of the BamHI (dephosphorylated)-EcoRI pUCC3d fragment, the BglII (desphosphorylated)-EcoRI pUCC3d fragment and the BglII-BamHI pUCC3d fragment. The SpeI fragment of pUCsgpM2C3d3 containing the sgpM2C3d3 fusion sequence was then inserted behind the polyhedrin promoter by exchange with the SpeI-XbaI fragment of the baculovirus transfer vector pACGP67A. The resulting transfer vector, called pACsgpM2C3d3, was then used to generate recombinant AcNPV/sgpM2C3d3 baculovirus by calcium phosphate cotransfection of Sf9 insect cells with BaculoGold baculovirus DNA (PharMingen, San Diego, CA, USA), following the procedure as described in King and Possee (1992). The presence of the sgpM2C3d3 fusion sequence behind the polyhedrin promoter in the genome of the corresponding recombinant AcNPV/sgpM2C3d3 baculovirus was confirmed by PCR analysis.

20

#### 16. Expression of secreted M2C3d3 by Sf9 insect cells

Log-phase Sf9 insect cells were inoculated with recombinant AcNPV/sgpM2C3d3 baculovirus at high multiplicity of infection ( $> 10$ ). Cells were subsequently transferred to serum-free TC100 medium and further incubated for 48 h before harvesting the supernatant. Proteins were precipitated by adding an equal volume of acetone (preequilibrated at  $-20^{\circ}\text{C}$ ) and subsequently analyzed by Western blotting.

30

In a preferred construction, three or more copies of the C3d protein are preceded by the extracellular domain of the M2 protein.

#### 17. Passive immunisation

35

The survival is shown in figure 28. In both control groups only one mouse out of 12 survived the lethal influenza challenge, while 11 out of 12 mice immunized with  $3 \times 10^6$  pg IM2HBcm or all passively

immunized mice were protected. This experiment demonstrates that anti-M2 antibodies produced during the vaccination account for the observed protection.

#### 5 18. DNA vaccination

Table 3 shows the results of a DNA vaccination experiment in which 12 mice injected with 3 x 100 µg pCIM2 were compared with a control group injected three times with 100 µg pCDNA3 for the survival against a  
10 lethal challenge (5 LD<sub>50</sub>) with m.a. X47. A partial protection against a heterologous (immunising antigen = universal M2, challenge = A/PR/8/34 derived M2) influenza challenge could be demonstrated.

15 Table 3

vector	surviving mice/total number
pCDNA3 (control)	1/12
pCIM2 (complete <u>m2</u> gene)	7/12

20

#### 19. Expression in HEKT cells

The expression level of the complete M2 protein is too low to be detected, in the soluble fraction and in the pellet (see figure 30). It is possible that the  
25 expression is kept low due to the ion channel activity of the M2 protein, which can be toxic for the HEKT cells. The two fusion proteins, IM2HBcm and IP3M2HBcm however are well expressed. This experiment demonstrates that the vectors used in the DNA vaccination studies can express  
30 the protein, except maybe for pCIM2.

#### 20. Analysis of the serum

A specific antibody response directed towards the extracellular part of the M2 protein could be  
35 demonstrated, although this response is low. In panel B from figure 31 pCIM2 is compared to the control vector.

In this ELISA M2 protein expressed in insect cells was used as coating (see Materials and Methods). A specific anti-M2 response could be demonstrated, especially after the third immunization. The higher anti-M2 response with 5 pCIM2 can be due to additional epitopes located in the cytoplasmic domain of the M2 protein.

## DISCUSSION

The present document describes several systems for the presentation of the highly conserved extracellular part of the influenza A virus M2 protein to the immune system. The M2 fragment was fused to the amino terminus of the carrier protein in order to retain a free N-terminus of the M2-domain and in this way mimic the wild type structure of the M2 protein. The first fusion protein, M2 linked to human  $\beta$ 2-microglobulin (IPM2hB2Mm), was used to produce monoclonal antibodies. A second fusion protein, M2 linked to Hepatitis B core protein (IPM2HBcm) was used for vaccination studies. Both proteins could also be used in the detection of a specific antibody response against the extracellular part of the M2 protein, since a correction has to be made for antibodies directed against the carrier protein, which are also produced during the immunization process.

The vaccination studies with IPM2HBcm showed that the administered dose in the range that was used, was apparently not a very critical parameter for obtaining protection, as a dose ranging from 5 to 50  $\mu$ g protected the mice, although the immunized mice still showed a high morbidity. This may have been due to the high dose of virus (5 LD<sub>50</sub>) that was used for the challenge in order to obtain a clear-cut result for the degree of protection. In a natural influenza infection the number of infecting virus particles is much lower, so that it can be assumed that the morbidity would decrease accordingly.

Analysis of the serum of immunized mice showed a substantial antibody response towards the extracellular

part of the M2 protein, especially after viral challenge. This latter, high response can be due to another way of administration, intraperitoneal versus intranasal. Or it can be explained on the basis of a more complete defense  
5 mechanism against the incoming virus.

Slepushkin et al. (1995) described a vaccination strategy, based on a membrane extract containing the natural complete M2 protein for homologous and heterologous virus challenge. But they used a very  
10 strong adjuvant, incomplete Freund's, which is not appropriate for medical use.

In contrast, the M2 extracellular domain fusions of the invention described here can be obtained in a pure form (at least 95% purity), and can be  
15 administered in combination with safe adjuvants. A high degree of protection was obtained, despite the fact that the challenge was fairly severe. In view of the almost invariant sequence of the M2 extracellular domain (see table 1 which shows an overview of the amino acid  
20 sequences of the extracellular domain of the influenza A M2 protein) it may be expected that the protection achieved will be similar against all human influenza A strains known so far.

The vaccine may be further improved by the  
25 inclusion of an influenza specific T helper epitope as well as a CTL epitope into the fusion protein, for example internally or linked to the C-terminus of the Hepatitis B core protein. Other immunization routes are possible as well, for example intraperitoneal versus  
30 intranasal.

Besides the gram negative organism, E. coli, also L. lactis was used, a gram positive organism, for the expression of the M2HBcm fusion proteins. In L. lactis it is not necessary to purify the expressed fusion  
35 protein. The bacteria can be administered directly either intranasally or orally.

A third promising carrier protein is also described, namely the third complement protein fragment d

00000-94086460



(C3d) (Dempsey et al., 1996). In a preferred construction, three copies of the C3d protein are preceded by the extracellular domain of the M2 protein. This M2C3d3 fusion protein can be expressed either in an intracellular form, anchored in the cell wall or secreted into the growth medium, by genetic fusion to appropriate regulatory sequences.

000000-000000

## REFERENCES

- Allen et al. (1980) *Virology* 107, 548 - 551
- Baez et al. (1980) *J. Infect. dis.* 141, 362-365
- Belshe et al. (1988) *J. Virol.* 62, 1508 - 1512
- Birnboim and Doly (1979) *N.A.R.* 7, 1513 - 1523
- Black et al. (1993a) *J. Gen. Virol.* 74, 143-146
- Black et al. (1993b) *J. Gen. Virol.* 74, 1673-1677
- Borisova et al. (1989) *FEBS Lett.* 259, 121 - 124
- Casadaban and Cohen (1980) *J. Mol. Biol.* 138, 179 - 207
- Clarke et al. (1987) *Nature* 330, 381 - 384
- Cohen and Richmond (1982) *Nature* 296, 677 - 678
- Cox et al. (1988) *Virology* 167, 554 - 567
- Dempsey et al. (1996) *Science* 271, 348 - 350
- Deng and Nickolov (1992) *Anal. Biochem.* 200, 81 - 88
- Gasson (1983) *J. Bact.* 154, 1 - 9
- Gillis et al. (1978) *J. Immunol.* 120, 2027 - 2032
- Hirst (1941) *Science* 94, 22 - 23
- Holsinger and Lamb (1991) *Virology* 183, 32 - 43
- Kahn et al. (1979) *Methods Enzymol.* 68, 268 - 280
- Kendal et al. (1982) *Concepts and procedures for laboratory-based influenza surveillance.* p. B7 - B1 2, B1 7 - B1 9
- King and Possee (1992) *The Baculovirus Expression System.* Chapman & Hall, University Press, Cambridge, UK
- Klimov et al. (1992) *Virology* 186, 795 - 797
- Köhler and Milstein (1975) *Nature* 256, 495 - 497
- Laemmli (1970) *Nature* 227, 680 - 685
- Lamb and Lai (1981) *Virology* 112, 746 - 751
- Lamb et al. (1981) *Proc. Natl. Acad. Sci. USA* 78, 4170 - 4174
- Lamb et al. (1985) *Cell* 40, 627 - 633
- Levi and Arnon (1996) *Vaccine* 14, 85 - 92
- Markushin et al. (1988) *Virus Res.* 10, 263 - 272
- Miller (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, p. 431
- Min Jou et al. (1980) *Cell* 19, 683-696
- Nakamaye and Eckstein (1986) *N.A.R.* 14, 9679 - 9698

- Nassal (1988) Gene 66, 279 - 294
- Neu and Heppel (1965) J. Biol. Chem. 240, 3685 - 3692
- Ortin et al. (1983) Gene 23, 233 - 239
- Parker and Wiley (1989) Gene 83, 117 - 124
- Remaut et al. (1981) Gene 15, 81 - 93
- Remaut et al. (1983a) N.A.R. 11, 4677 - 4688
- Remaut et al. (1983b) Gene 22, 103 - 113
- Schöder et al. (1992) J. Virol. 66, 106 - 114
- Slepushkin et al. (1995) Vaccine 13, 1399 - 1402
- Stanssens et al. (1989) N.A. R. 17, 4441 - 4454
- Steidler et al. (1994) Biotechn. Bioeng. 44, 1074 - 1082
- Steidler et al. (1995) Appl. Environ. Microbiol. 61, 1627 - 1629
- Steidler et al. (1996) NATO ASI Series H 98 p 63 - 79.
- eds. Bozoglu, T.F. and Ray, B. Springer, Berlin
- Struhl (1985) Biotechniques 3, 452 - 453
- Sugrue et al. (1990) Virology 179, 51 - 56
- Sugrue and Hay (1991) Virology 180, 617 - 624
- Treanor et al. (1990) J. Virol. 64, 1375 - 1377
- van Asseldonk et al. (1990) Gene 95, 155 - 160
- van de Guchte et al. (1992) FEMS Microbiol. Rev. 88, 73 - 92
- Van Snick et al. (1986) Proc. Natl. Acad. Sci. USA 83, 9679
- Vogelstein and Gillespie (1979) Proc. Natl. Acad. Sci. USA 76, 615 - 619
- Wells et al. (1993) J. Appl. Bact. 74, 629 - 636
- Wells and Schofield (1996) NATO ASI Series H 98 p 37 - 62. eds. Bozoglu, T.F. and Ray, B. Springer, Berlin
- Winter and Fields (1980) N.A.R. 8, 1965 - 1974
- Zebedee and Lamb (1988) J. Virol. 62, 2762 - 2772
- Zebedee and Lamb (1989) N.A.R. 17, 2870
- Zell and Fritz (1987) EMBO J. 6, 1809 - 1815

004020-94080460